Type VIII Collagen Has a Restricted Distribution in Specialized Extracellular Matrices

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Abstract. A pepsin-resistant triple helical domain (chain 50,000 M_r) of type VIII collagen was isolated from bovine corneal Descemet's membrane and used as an immunogen for the production of mAbs. An antibody was selected for biochemical and tissue immunofluorescence studies which reacted both with Descemet's membrane and with type VIII collagen 50,000-M_r polypeptides by competition ELISA and immunoblotting. This antibody exhibited no cross-reactivity with collagen types I-VI by competition ELISA.

The mAb specifically precipitated a high molecular mass component of type VIII collagen (EC2, of chain 125,000 M_r) from the culture medium of subconfluent bovine corneal endothelial cells metabolically labeled for 24 h. In contrast, confluent cells in the presence of FCS and isotope for 7 d secreted a collagenous component of chain 60,000 M_r that did not react with the anti-type VIII collagen IgG. Type VIII collagen therefore appears to be synthesized as a discontinuous triple helical molecule with a predominant chain 125,000 M_r by subconfluent, proliferating cells in culture.

Immunofluorescence studies with the mAb showed that type VIII collagen was deposited as fibrils in the extracellular matrix of corneal endothelial cells. In the fetal calf, type VIII collagen was absent from basement membranes and was found in a limited number of tissues. In addition to the linear staining pattern observed in the Descemet's membrane, type VIII collagen was found in highly fibrillar arrays in the ocular sclera, in the meninges surrounding brain, spinal cord, and optic nerve, and in periosteum and perichondrium. Fine fibrils were evident in the white matter of spinal cord, whereas a more generalized staining was apparent in the matrices of cartilage and bone. Despite attempts to unmask the epitope, type VIII collagen was not found in aorta, kidney, lung, liver, skin, and ligament. We conclude that this unusual collagen is a component of certain specialized extracellular matrices, several of which are derived from the neural crest.

TYPE VIII collagen was first isolated from the culture medium of metabolically labeled bovine aortic endothelial cells (22, 24). This collagen was termed EC (endothelial collagen) and a survey of its distribution showed that it was secreted in limited amounts by most cultured bovine vascular and corneal endothelial cells and by human fibroblasts (21). Type VIII collagen was also produced in somewhat larger amounts by cell lines derived from a human astrocytoma (1), Ewing's sarcoma (of the femur), and adenocarcinomas metastatic to bone and pelvis (21). Since most studies on type VIII collagen to date have been carried out in cell culture systems or with Descemet's membrane (DM[1]; a specialized extracellular matrix [ECM] subjacent to the corneal endothelium), the general distribution of this collagen in tissues is not known.

Studies in vitro have suggested that native type VIII collagen consists of pepsin-resistant, triple-helical collagenous domains (chain 50.000 M_r) linked in tandem by noncollagenous domains of M_r < 10,000 that compose the major secreted products of chain 177,000 M_r (EC1) and 125,000 M_r (EC2; see reference 20 for a review of this "cassette" model). Recently, pepsin-resistant collagenous fragments (M_r = 50 kD) isolated from bovine corneal DM have been shown to be derived from type VIII collagen (11). Rabbit antisera specifically reactive with the pepsin-resistant fragments extracted from tissue have been used to demonstrate the immunological identity of the tissue fragments with the type VIII chain, EC2, synthesized by bovine corneal endothelial (BCE) cells. These polyclonal antibodies have also localized type VIII collagen to DM. Benya and Padilla (3), using a

1. *Abbreviations used in this paper*: BCE, bovine corneal endothelial; DM, Descemet's membrane; ECM, extracellular matrix; RCE, rabbit corneal endothelial.

different procedure for the isolation of type VIII collagen from the culture medium of endothelial cells, have proposed an alternate model in which type VIII collagen was comprised of a triple helix of chain 61,000 M_r.

In the present study we have generated mAbs specific for the pepsin-resistant, triple-helical domains of type VIII collagen. We present evidence that the secreted form of type VIII collagen is EC2 of chain 125,000 M_r, and we show that type VIII collagen is located in the ECM of BCE cells in vitro. The tissue distribution of type VIII collagen in fetal bovine tissues was found to be restricted to specialized connective tissue matrices, including perichondrium, periosteum, and cartilage. In addition, several structures of neural crest origin, such as calvarium, DM, ocular sclera, and meningial coverings contained significant amounts of this unusual collagen.

Materials and Methods

Isolation of Collagens from DM and Perichondrium

Frozen adult bovine eyes were obtained from Pel-Freeze Biologicals (Rogers, AR). Collagens were isolated by a short pepsin digestion of DM, followed by salt fractionation as previously described by Kapoor et al (11). Bovine trachea, obtained from a local slaughterhouse, was rinsed and transported at 0°C in PBS containing 0.1 M ε-amino caproic acid, 0.1 M EDTA, 5 mM *N*-ethylmaleimide, 5 mM benzamidine hydrochloride, and 1 mM $PhCH_2$-SO_2F. Loose connective tissue adhering to the trachea was removed, and the perichondrium was peeled from the underlying cartilage. The perichondrium was sliced into small pieces (~1 mm^3), washed with detergent/DNase (4), and digested with pepsin. The collagenous proteins were subsequently fractionated by differential precipitation in NaCl (11).

Cell Culture and Metabolic Labeling

Primary cultures of BCE cells were prepared by modifications of the method described by MacCallum et al. (18). Fresh adult bovine eyes from a local slaughterhouse were transported in cold PBS containing 200 μg/ml each of streptomycin and penicillin G. 6–10 eyes were used for each primary culture. After a thorough wash under running tap water, the corneal side of each orbit was liberally rinsed with 70% ethanol followed immediately by PBS. The corneas were then excised and sequentially rinsed in four dishes containing serum-free DME with 5 μg/ml Amphotericin B, and 50 μg/ml Gentamycin in addition to penicillin/streptomycin. Corneas were placed, endothelial side up, in a petri dish. 15-mm rings cut from stainless steel pipe and dipped in sterile silicon grease were set firmly onto each cornea to create a well for trypsinizing the cells. The endothelium was rinsed twice with 0.02% EDTA in PBS and incubated at 37°C in a solution of 2.5% trypsin/1% EDTA (Gibco, Grand Island, NY) for 5 min. Cells were removed by gently pipetting up and down and rinsing with DME containing 16% FCS and antibiotics. The cell suspension was plated into a 35-mm tissue culture dish and allowed to attach for 3–4 h before the media were changed. Cultures were maintained in DME containing 16% serum and passaged at a 1:3 subcultivation ratio. The results presented are from subcultures in the third and fifth passage.

Metabolic labeling of cells with L-[2,3,4,5-^{3}H]proline (100 Ci/mol; Amersham Corp., Arlington Heights, IL) was carried out on nearly confluent cells as described by Sage et al. (22). Proteins including type VIII collagen were subsequently isolated from the culture medium by ammonium sulfate precipitation and DEAE-cellulose chromatography (24).

Confluent cultures of BCE and rabbit corneal endothelial cells (RCE) were labeled in DME containing 2% FBS, 25 μg/ml sodium ascorbate, 62.5 μg/ml β-aminopropionitrile, and 50 μCi/ml L-[2,3-^{3}H]proline (35 Ci/mol). The medium was collected daily and replaced with fresh medium (supplemented as above) for 7 d. This postculture medium was adjusted to the following buffer and protease inhibitor concentrations: 50 mM Tris–HCl, pH 8, 20 mM *N*-ethylmaleimide, 10 mM ε-aminocaproic acid, 10 mM benzamidine hydrochloride, 10 mM EDTA, 1 mM $PhCH_2SO_2F$, 0.23 TIU/ml aprotinin (Sigma A-6012), and 10 μg/ml each of leupeptin, antipain, and chymostatin (Sigma Chemical Co., St. Louis, MO). After centrifugation to remove cellular debris, the medium was stored at −20°C. The type VIII collagen was subsequently isolated from the pooled media by a combination of ultrafiltration, sucrose density gradient sedimentation, salt fractionation, and ion-exchange chromatography, exactly as described by Benya and Padilla (3).

Production of mAbs

A.Sw/J mice (Jackson Laboratory, Bar Harbor, ME) were immunized with the pepsin-resistant fragments of type VIII collagen, and their splenic lymphocytes were fused with the myeloma cell line P3-NS1/1-Ag4-1 (14) according to previously described methods (25, 26). Hybridomas were screened for the production of specific antibody by ELISA (5) and by indirect immunofluorescence. Antibody was collected from selected hybridoma clones either as spent culture supernate or as ascites from Balb/c × ASw hybrid mice. These animals had been primed with pristane and injected with a minimum of 10^7 hybridoma cells.

Immunoblotting and ELISA

SDS-PAGE was performed according to Laemmli (16) on polyacrylamide slab gels containing 0.5 M urea, and proteins were transferred to nitrocellulose sheets. Immunoblotting was carried out according to Towbin et al. (30), as modified by Kapoor et al. (11). ELISA was performed on microtiter plates coated with 1–5 μg antigen. Inhibition of binding was tested by mixing antibody, diluted to the concentration that produced one-half the maximal binding, with inhibitor at various concentrations for 12 h at 4°C. Digestion of the antigen by bacterial collagenase before ELISA was performed as described (25).

Immunoprecipitation and Affinity Chromatography

Culture medium proteins were obtained from metabolically labeled BCE cells by ammonium sulfate precipitation and DEAE-cellulose chromatography. The unbound chromatographic fraction was dialyzed against 0.1 M acetic acid at 4°C and lyophilized. It was then dissolved in 2 ml of PBS, pH 7.4, and divided into two equal parts. To one part, 100 μl of polyclonal antibodies, affinity-purified against the pepsin-resistant fragments of type VIII collagen (11), was added, and to the other part 100 μl of the mAbs was added. The mixtures were shaken gently overnight at 4°C. Subsequently, 100 μl of Protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), washed in a solution of BSA to block nonspecific binding and resuspended in 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl at pH 7.4, was added to each tube. The mixtures were shaken gently at room temperature for 2–3 h and subsequently centrifuged (microfuge). The pellet was washed with the same buffer and centrifuged for 3 min. This procedure was repeated five times. Finally, the pellet was dissolved in 50 μl of Laemmli buffer (16), heated at 100°C for 2–3 min, and centrifuged for 3 min. The supernate was analyzed by electrophoresis on an 8% polyacrylamide gel.

The mAb was coupled to CNBr-activated Sepharose according to the manufacturer's instructions. Proteins, isolated from the culture medium of metabolically labeled BCE cells, were dissolved in 0.5–1.0 ml of PBS and equilibrated with the affinity resin for 30 min at 4°C. The eluate was rechromatographed three times, and the column was washed extensively with PBS until the radioactive counts returned to background level. The bound protein was then eluted with 0.02 M HCl/glycine (pH 2.2). A peak of radioactivity was collected, dialyzed against 0.1 M acetic acid at 4°C, and lyophilized.

Immunofluorescence Microscopy

Although several calves representing a broad spectrum of fetal age were examined, tissue sections used in this study were taken from a single fetal calf that measured ~1 foot 5 inches from crown to rump. The animal was obtained at a local slaughterhouse shortly after removal from the mother. The estimated age of this fetus was 140 d, a stage of development before the onset of extensive ossification. Tissues were dissected and frozen in hexane in liquid nitrogen. Eight micron sections were cut using a histostat microtome (American Optics, Buffalo, NY) at −28°C. Sections were air dried for 30 min and fixed in cold acetone for 10 min. mAb (ascites) diluted 1:100 with PBS was incubated with the sections for 3 h at room temperature. After a wash with PBS, FITC-conjugated anti-mouse IgG (Sigma Chemical Co.), diluted 1:50 with PBS, was incubated with the sections for 30 min. Slides were then washed several times with PBS, and the sections were covered with 90% glycerol in PBS. Photographs were taken with a Zeiss Photomicroscope III.

Some tissue sections were treated with hyaluronidase (Worthington Biochemicals, Freehold, NJ), at a concentration of 8,000 U/ml in 0.1 M phosphate buffer, pH 5.3, for 20 min at room temperature. Alternatively, sections were exposed to pepsin (0.1 mg/ml; Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) in 0.1 M acetic acid for 10 min at room temperature, or to 0.1 M acetic acid at room temperature for 30 min. The sections were washed with PBS and prepared for immunofluorescence microscopy.

BCE cells were carefully isolated with 0.025% trypsin (Sigma Chemical Co.) from freshly dissected eyes. Cells were grown on chamber slides in DME, supplemented with 10% FBS and penicillin/streptomycin (Gibco). Cells were washed with PBS, fixed in cold acetone for 10 min, and stained as described above. Alternatively, for experiments with rabbit polyclonal antibodies, cells were grown on glass coverslips and fixed in buffered paraformaldehyde (3%) as described by Sage et al. (23), except that cells were not permeabilized before incubation with antibodies. For staining unfixed cells, the cells were preincubated in medium without serum for 30 min at 37°C, washed twice with PBS for 10 min each, and incubated with primary antibody for 90 min at room temperature. The cells were then washed with serum-free medium for 10 min, rinsed twice with PBS, and incubated with FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) for 30 min. Cells were washed twice with PBS and were then fixed in 3% paraformaldehyde for 30 min. The coverslips were washed three times with PBS and mounted on glass slides. To analyze the extracellular matrix, the cells were removed from the coverslips by treatment with 0.1% Triton X-100 for 2–5 min at 37°C. Polyclonal antibodies affinity-purified against bovine type III procollagen have been described by Sage et al. (23).

Results

Characterization of Anti-type VIII Collagen mAb

Hybridoma culture wells were first screened by ELISA for binding to the original immunogen, a native preparation containing the pepsin-resistant domains of bovine type V and type VIII collagens. Supernatant media which were positive by ELISA were then tested by immunofluorescence. Two wells contained antibody that stained DM. Further analysis by immunoblotting showed that one of the clones (an IgG) secreted antibody which reacted specifically with the 50-kD pepsin-resistant fragments of type VIII collagen. This clone was selected for the present study.

ELISA reactivity with the pepsin-resistant fragments of type VIII collagen could be inhibited by prior incubation of the fragments with a dilution of ascites mAb, but this binding could not be inhibited by incubation with types I, III, IV, V, and VI collagens (Fig. 1). The epitope on type VIII (50-kD fragments) was, however, sensitive to bacterial collagenase (data not shown). This antibody also reacted with both chicken and human type VIII collagen.

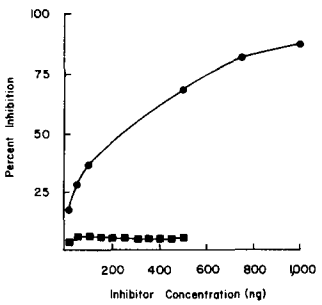

Figure 1. Characterization of monoclonal antibody by ELISA. Microtiter plates were coated with 5 μg of 50,000-M_r collagenous fragments of type VIII collagen dissolved in 0.1 M Na_2CO_3 buffer, pH 8. Competition ELISA with mAbs (1:10,000) was carried out by incubation with varying concentrations of antigens at 4°C for 24 h. (●) 50,000-M_r fragments of type VIII collagen; (■) collagen types I, III, IV, V, and VI.

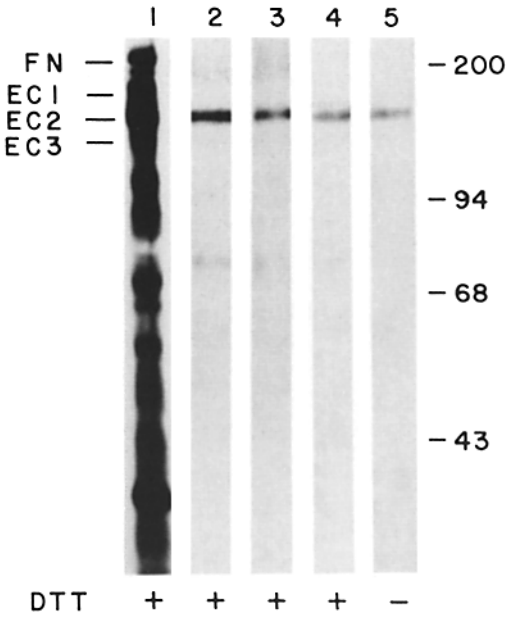

Figure 2. Immunoisolation of type VIII collagen by monoclonal and affinity purified polyclonal anti-50,000-M_r IgG. Type VIII collagen was isolated from the culture medium of [^{3}H]proline-labeled BCE cells. Proteins that were not bound by DEAE-cellulose were dialyzed against 0.5 M acetic acid at 4°C and lyophilized. Aliquots of this material were immunoprecipitated with monoclonal and polyclonal antibodies and were subsequently analyzed by SDS-PAGE on an 8% gel. Lane *1*, total fraction; lane *2*, immunoprecipitation with polyclonal anti-50,000-M_r IgG (band represents 11.1% of input cpm); lane *3*, immunoprecipitation with monoclonal anti-50,000-M_r IgG (3.6% of input cpm). Alternatively, BCE culture medium proteins were chromatographed over an affinity column of monoclonal anti-50,000-M_r IgG coupled to Sepharose, and the bound proteins were eluted with 0.02 M glycine, pH 2.2 (lane *4*). Lane *5*, lane *4* before reduction with dithiothreitol (DTT). Molecular mass standards of globular proteins shown on the right are 200,000, 94,000, 68,000, and 43,000 D. EC1, 2, and 3 denote the chains of type VIII collagen, with M_r of 180,000, 125,000, and 100,000, respectively.

Immunologic and Biosynthetic Studies: Evidence for the Cassette Model

Proteins were fractionated from the culture medium of metabolically labeled BCE cells. Analysis by SDS-PAGE revealed the EC1, EC2, and EC3 chains of type VIII collagen (Fig. 2, lane *1*); these components were degraded by bacterial collagenase (results not shown). EC2 was specifically precipitated by the mAb and by affinity-purified polyclonal antibodies against the 50,000-M_r fragments of type VIII collagen (Fig. 2, lanes *2* and *3*). This finding was confirmed by affinity purification of type VIII collagen from the culture medium of BCE cells by adsorption to mAb-Sepharose. The proteins bound to the affinity column were eluted in a peak that represented 1.0% of the original counts applied to the column. SDS-PAGE showed a single band corresponding to EC2 which was unaffected by disulfide bond reduction (Fig. 2, lanes *4* and *5*).

Since both preparations of antibodies precipitated a type VIII collagen chain of $M_r > 60{,}000$, we examined the effect of culture conditions on the initial molecular size of the secreted protein. Type VIII collagen was purified from the

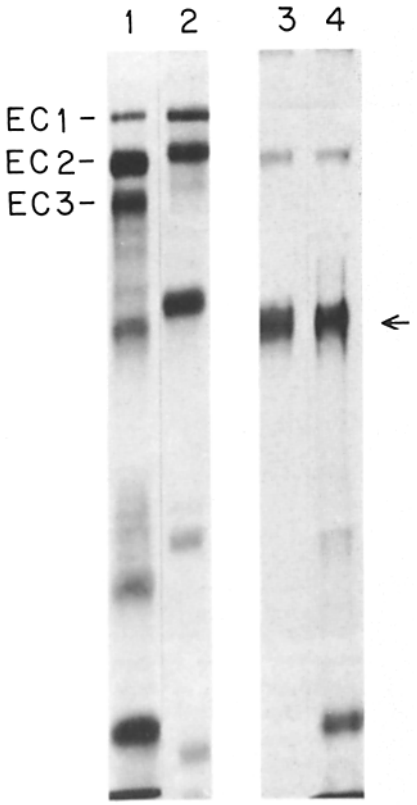

Figure 3. Analysis of type VIII collagen isolated from the culture medium of BCE and RCE cells. Subconfluent and confluent BCE and RCE cells were incubated with [^{3}H]proline for 24 h and 7 d. Proteins were resolved on an 8% SDS gel under reducing conditions and were visualized by fluorescence autoradiography. Lanes *1* and *2*, subconfluent BCE and RCE cells, respectively, labeled for 24 h; lanes *3* and *4*, confluent BCE and RCE cells, respectively, labeled for 7 d. The arrow indicates a protein of ~60,000 M_r that accumulated in the cell culture medium during the 7-d labeling period.

culture medium of subconfluent and fully confluent RCE and BCE cells. EC1, EC2, and EC3 could be demonstrated in the culture medium of subconfluent BCE cells after a 24-h labeling period (Fig. 3, lane *1*). EC1 and EC2 were also present in the culture medium of subconfluent RCE cells (Fig. 3, lane *2*). Labeling of fully confluent cells for longer periods (7 d) resulted in a reduction of the amount of EC2, and a loss of EC1 and EC3, from the culture media of both RCE and BCE cells (Fig. 3, lanes *3* and *4*). In addition, a protein with an approximate M_r of 60,000 was apparent in the culture media of these cells (Fig. 3, lanes *1–4*, *arrow*). This protein did not cross react with anti-50,000-M_r IgG by Western blotting (results not shown). Furthermore, in all the experiments in which we have exposed BCE culture media to monoclonal or polyclonal anti-type VIII collagen IgG, we have not observed reactivity with a component of 60,000 M_r. We therefore suggest that under conditions of prolonged culture in the presence of serum, corneal endothelial cells modulate collagen synthesis and switch to the production of the 60-kD collagen previously described by Benya and Padilla (3).

Immunofluorescence In Vitro

Immunofluorescence studies of BCE cells in culture showed that type VIII collagen accumulated in the extracellular matrix as a function of time in culture. After 12 d, fibrillar staining was seen in various regions of the slide (Fig. 4 *A*). After 34 d in culture, immunofluorescent fibers were more abundant (Fig. 4 *B*). Accumulation type VIII collagen in the cell layer as a function of time provides an explanation for its depletion from the culture medium. The fibrillar staining patterns of type VIII collagen, demonstrated for comparative purposes with affinity-purified polyclonal antibodies raised against the 50,000-M_r fragments in Fig. 4, *D* and *F*, were less dense than those observed for type III collagen (Fig. 4, *C* and *E*). Similar patterns were obtained whether the cells were fixed in paraformaldehyde (not shown), were first stained live and then fixed in paraformaldehyde (Fig. 4, *C* and *D*), or were first removed with 0.1% Triton X-100 and the underlying matrix stained (Fig. 4, *E* and *F*). No staining of the cells or matrix was seen when preimmune serum was used (data not shown).

Distribution of Type VIII Collagen In Vivo

Tissues from a single calf (estimated age was 140 d) were selected for immunofluorescence examination using the anti-type VIII collagen mAb. There was linear staining of DM in the cornea, but no fluorescence in the stroma or Bowman's membrane (Fig. 5 *a*). In the surrounding sclera (Fig. 5 *c*), type VIII appeared highly fibrillar and resembled fibrillin, a constitutive structural element of extracellular microfibrils (26). Staining with an mAb specific for fibrillin is shown for comparison, in Fig. 5, *b* and *d*. Type VIII collagen immunofluorescence also clearly defined the corneal–scleral junction (data not shown).

While the staining pattern of type VIII collagen in DM and sclera seemed similar to that of fibrillin, the distribution of type VIII was highly restricted. Bundles of fibrillin gave a dispersed fibrillar staining in the corneal stroma as well as a linear staining of DM (Fig. 5 *b*), and there was significant staining of the sclera (Fig. 5 *d*). Fibrillin was present as a major component of aorta (Fig. 5 *f*) and of nuchal ligament (Fig. 5 *h*), while type VIII appeared to be absent from aorta (Fig. 5 *e*) and nuchal ligament (Fig. 5 *g*). With anti-type VIII antibodies there was no immunofluorescence of aortic intima or media despite attempts to "unmask" the tissue by treatment with hyaluronidase, pepsin, or acetic acid. Other tissues which were also negative for type VIII collagen included lens capsule, kidney, lung, skin, and liver. From these results we conclude that type VIII collagen is not a structural component of basement membranes, nor is it preferentially associated with the vasculature.

Type VIII collagen was present in cartilage and calvarium, as shown in Fig. 6, *a–d*. Perichondrial immunofluorescence was observed around elastic cartilage, but fluorescence was not seen within the cartilage matrix (Fig. 6 *a*). Digestion with hyaluronidase was required to demonstrate the presence of type VIII collagen around chondrocytes (Fig. 6 *b*). This collagen was present in fibrillar arrays in the periosteum and dura mater (Fig. 6 *c*), as well as in the calvarium (Fig. 6 *d*). Type VIII was also localized to the perichondrium of cartilage at the epiphysis of the femur (Fig. 7 *a*) and to areas within the cartilage matrix close to the secondary center of ossification (Fig. 7 *b*). Hyaluronidase treatment was not necessary to demonstrate this immunofluorescence.

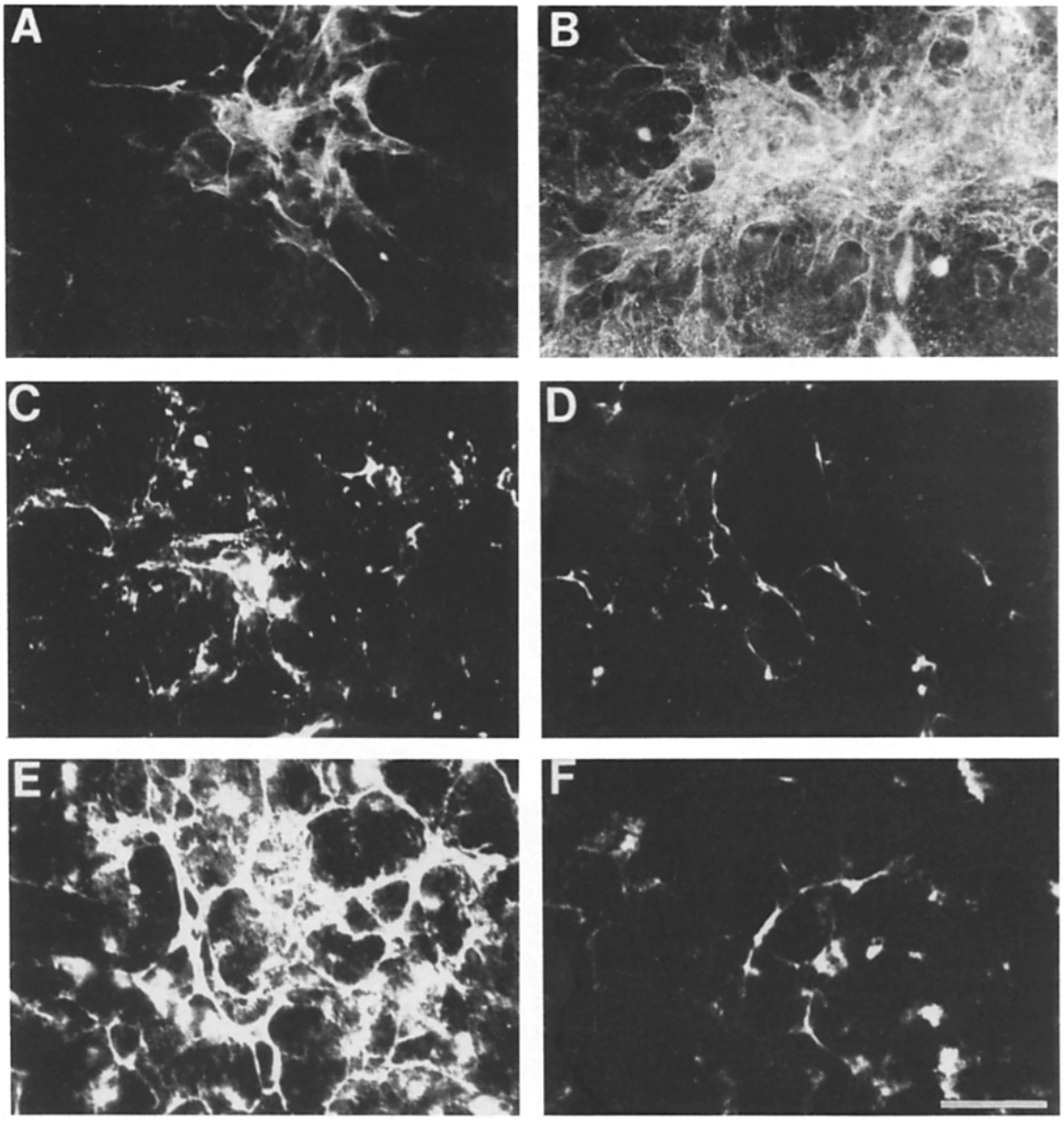

Figure 4. Immunofluorescence of BCE cells in vitro. Cells were grown on glass coverslips and exposed sequentially to monoclonal or affinity-purified polyclonal antibodies and FITC-conjugated goat anti-rabbit IgG. The ECM produced by these cells was also examined after removal of the cells by brief treatment with Triton X-100. After fixation in cold acetone for 10 min, cells in culture for (*A*) 12 and (*B*) 34 d were exposed to monoclonal anti-50,000-M_r polypeptide IgG. Unfixed cells were exposed to (*C*) anti-type III procollagen IgG and (*D*) anti-50,000-M_r IgG. The ECM was reacted with (*E*) anti-type III procollagen IgG and (*F*) anti-50,000-M_r IgG. Bar, 50 μm.

Since the immunofluorescence studies indicated that type VIII collagen was present in perichondrium, we compared the collagens from bovine perichondrium with those of bovine DM. Collagens from these tissues were extracted by brief pepsin digestion and were partially purified by precipitation with NaCl. Type V collagen and 50,000-M_r fragments were present in the extract of DM (Fig. 8, lane *1*), while the perichondrium contained only type II collagen by Coomassie Blue staining of the polyacrylamide gel (Fig. 8, lane *2*). Immunoblotting of these fractions with the mAb, however, showed a strong positive reaction with 50,000-M_r fragments in both the DM and perichondrium (Fig. 8, lanes *3* and *4*) and no reaction with collagen types V or II. Thus by biochemical and immunologic criteria, type VIII collagen was present in the perichondrium of fetal and adult cows.

The dural covering of the optic nerve was positive for type VIII collagen; however, the inner meningial lining was negative (Fig. 9 *a*). In the spinal cord, the meninges were stained with anti-type VIII mAb. Additional fine fibers were also demonstrated within the white matter, but not the gray matter, of the spinal cord (Fig. 9 *c*). In comparison, fibrillin was present throughout the meninges of the optic nerve (Fig. 9 *b*). Fibrillin was also apparent in the meninges around the spinal cord and in blood vessels within the spinal cord, but its distribution was afibrillar (Fig. 9 *d*).

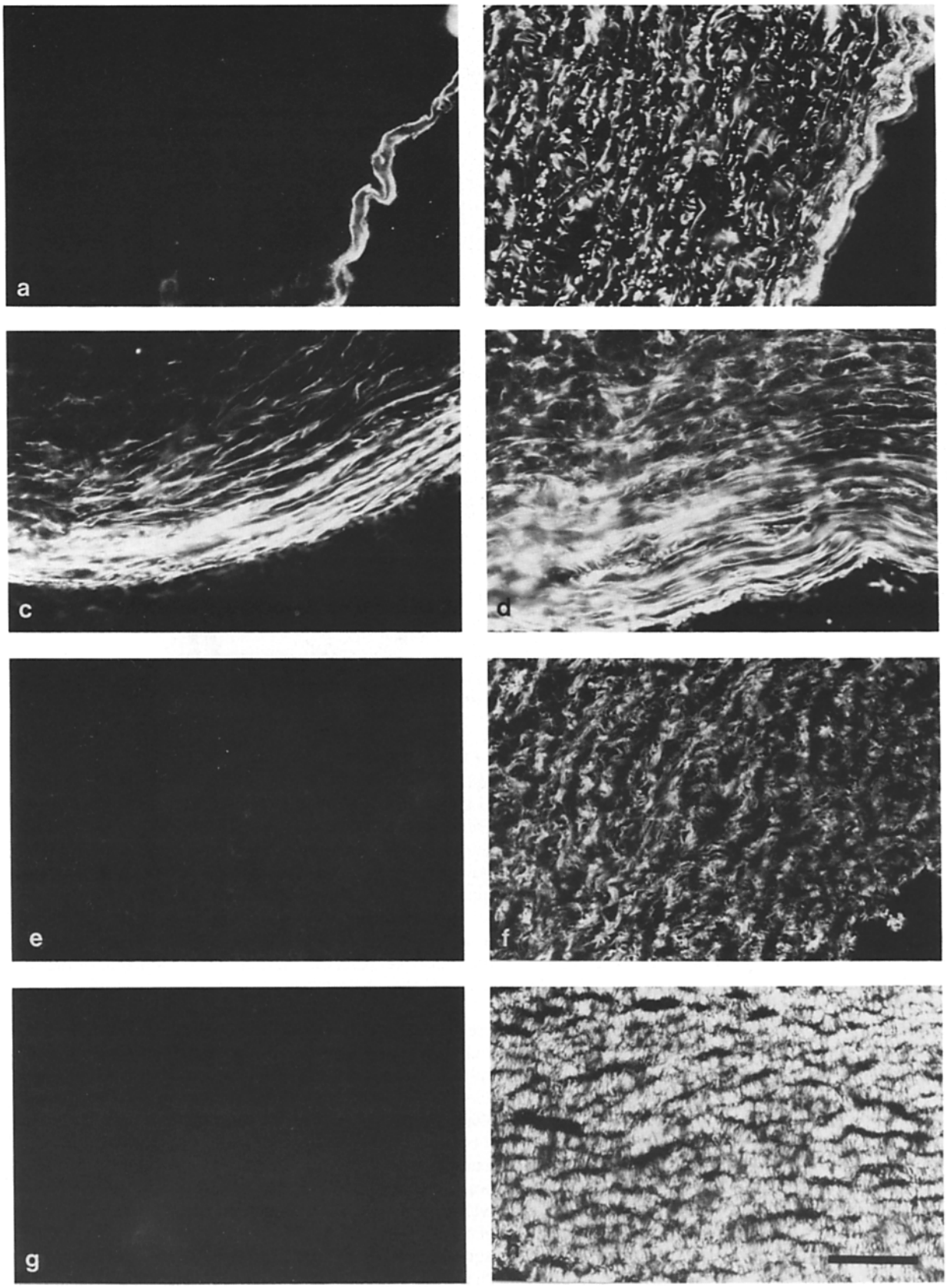

Figure 5. Distribution of type VIII collagen in fetal calf tissues: comparison to fibrillin. Frozen sections of fetal calf cornea (*a* and *b*), sclera (*c* and *d*), aorta (*e* and *f*), and nuchal ligament (*g* and *h*) were exposed sequentially to anti-type VIII collagen mAb (*a*, *c*, *e* and *g*) or anti-fibrillin mAb (*b*, *d*, *f*, and *h*), and FITC anti-mouse IgG. Bar, 50 μm.

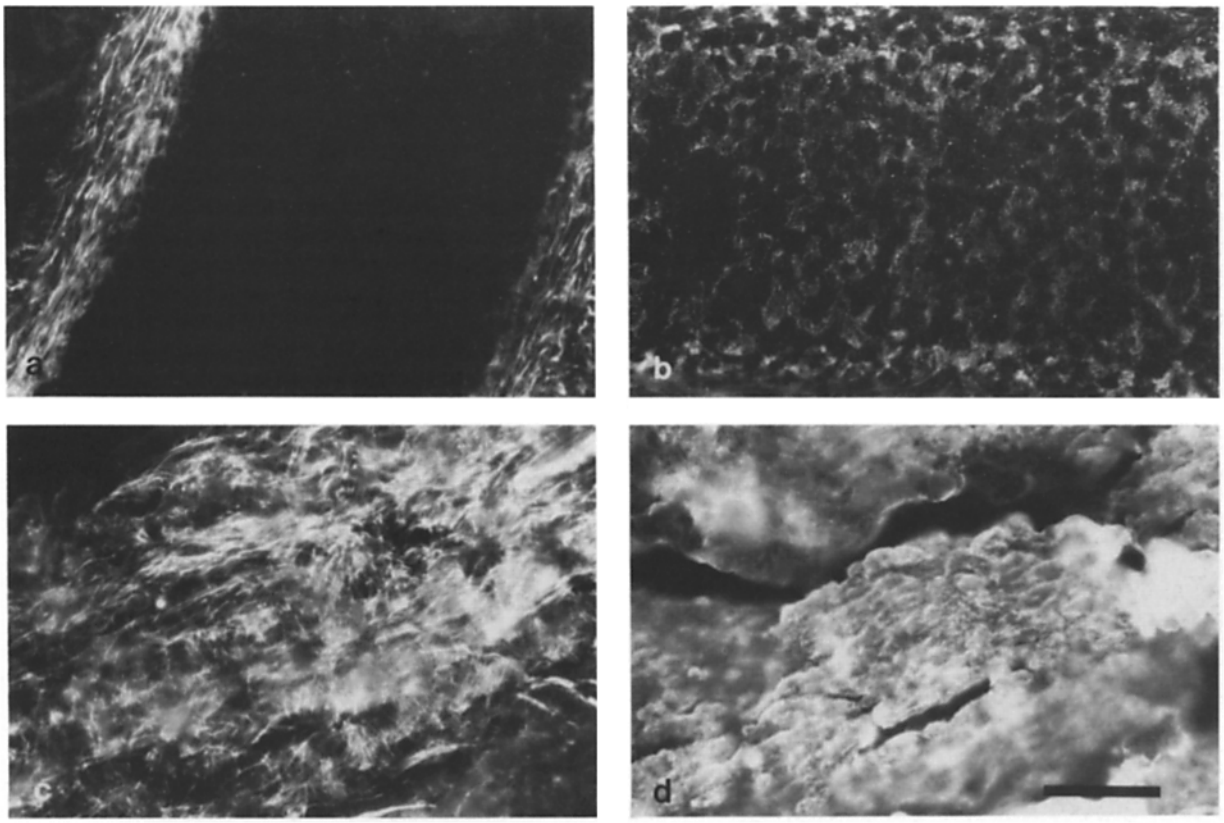

Figure 6. Distribution of type VIII collagen in cartilage and calvarium. Frozen sections of fetal calf ear cartilage (*a* and *b*), periosteum and dura mater of calvarium (*c*), and calvarial interstitium (*d*) were exposed sequentially to anti-type VIII collagen antibody and FITC anti-mouse IgG. *b* shows a similar section to *a* after treatment with hyaluronidase. Bar, 50 μm.

Discussion

We have used an mAb specific for pepsin-resistant fragments of type VIII collagen to identify this protein in a restricted number of tissues: DM of the cornea, the sclera, perichondrium and periosteum, cartilage matrix, bone, meninges of the central nervous system, and fine fibrils within the white matter of the spinal cord. In addition, we have used both polyclonal and monoclonal antibodies to identify EC2 (chain 125,000 M_r) as the principal molecular mass form of secreted type VIII collagen.

Sage et al. had proposed a model for type VIII collagen based on the secretion of EC1, EC2, and sometimes EC3 by subconfluent cells labeled for <24 h (22, 24). We now show that prolonged labeling resulted in a substantial reduction or loss of the EC components in RCE and BCE cell culture medium and accumulation of a 60,000-M_r protein that did not cross react with anti-50,000-M_r IgG. Moreover, anti-type VIII collagen antibodies specifically recognized EC2 in the culture medium of BCE cells labeled for <24 h. Based on pulse-chase data presented by Sage et al. (24), and the recognition of EC2 by polyclonal and monoclonal antibodies, it would appear that type VIII collagen is primarily synthesized in the form of EC2. In contrast, Benya and Padilla (3) studied confluent RCE cells labeled for prolonged periods in the presence of serum. These workers isolated a 61,000-M_r protein that they identified as type VIII collagen. We suggest that this lower molecular mass collagen differs from type VIII.

Our immunohistochemical survey with mAbs to type VIII collagen indicated a very restricted distribution for this protein. Although type VIII collagen is produced by several kinds of endothelia in vitro, we did not detect it by immunofluorescence in aorta nor in highly vascularized tissues like liver and lung, despite the use of standard procedures for unmasking epitopes on collagens (17). Since type VIII collagen was preferentially recovered from the culture medium of rapidly proliferating and/or migrating cells in vitro (20), it might only be expressed during the development of the vasculature or in the adult as a result of vascular injury.

The DM of the cornea is embryologically and ultrastructurally a unique ECM. Since a major portion of this specialized structure is synthesized by the corneal endothelium, the embryonic origin of this cellular population is of considerable interest. Although most studies have been performed in avian species (8, 9), it is now generally accepted that the DM in both birds and mammals is derived from a population of neural crest cells that invades the primary mesenchyme, a mesodermal layer surrounding the early optic cup. Collectively these cells form the secondary mesenchyme, the neural crest component of which, in a series of migrations, subsequently gives rise to corneal stroma, endothelium, and other ocular structures (see reference 2 for a review). Recent studies with mAbs have supported the hypothesis that the corneal endothelial cells are derived from early periocular vascular endothelium (6).

Electron microscopy has shown that the DM is comprised of fibers arranged in hexagonal lattices (10, 27). We speculate

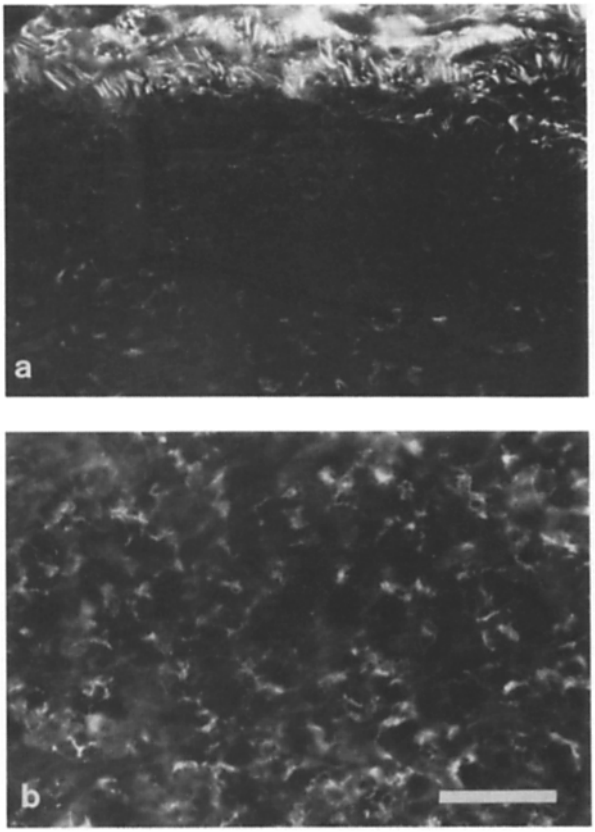

Figure 7. Occurrence of type VIII collagen in the matrix of cartilage. Frozen sections of fetal calf femur were reacted sequentially with anti-type VIII collagen antibody and FITC anti-mouse IgG. (*a*) Epiphysis including perichondrium; (*b*) cartilage matrix near secondary center of ossification. Bar, 50 μm.

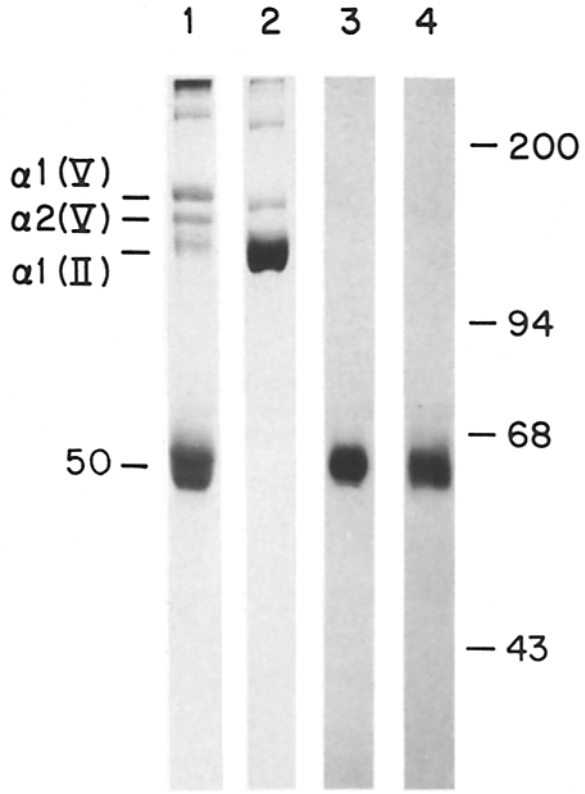

Figure 8. Analysis of collagens purified from the pepsin-treated bovine DM and perichondrium. Samples were resolved on 8% SDS-polyacrylamide gels and visualized by staining with Coomassie Blue (lanes *1* and *2*) or autoradiography (lane *3* and *4*). Lane *1*, bovine DM; lane *2*, perichondrium. The collagenous proteins were transferred from a polyacrylamide gel to nitrocellulose and immunoblotted with monoclonal anti-50,000-M_r IgG. Lane 3, bovine DM (antibody dilution was 1:100); lane *4*, bovine perichondrium (antibody dilution was 1:40). Protein molecular mass standards ($\times 10^{-3}$ D) are shown.

that type VIII collagen contributes to this fibrillar network, as Labermeier and Kenney (15) have claimed that a substantial proportion of the collagen in bovine DM is type VIII. We found that type VIII collagen was not codistributed with types I, III, V, and VI collagen in the corneal stroma but was restricted to the DM. The absence of type VIII collagen from the area of Bowman's membrane subjacent to the corneal epithelium, as well as from other BM, suggests that type VIII collagen is not immediately associated with type IV collagen of the lamina densa.

Type VIII collagen is also a component of cartilage matrix and perichondrium. The embryonic relationship of these tissues to DM is, however, not clear. The perichondrium is populated by osteoprogenitor cells contributing to fibrogenic and osteogenic layers, while DM is secreted by fibroblasts of the corneal stroma and by the corneal endothelium (8, 9, 12). Since the development of both cornea and endochondral bone is associated with the transient synthesis of type II collagen (31), it is possible that expression of the type II and type VIII genes is coregulated during embryogenesis. We are currently testing this hypothesis by examining embryonic chicks at different developmental stages.

Our results indicate that type VIII collagen is prevalent in mid-gestation fetal calf calvarium. The formation of cartilage and bone from a neural crest-derived mesenchyme is characteristic of skeletal tissues of the head. Studies in vitro have shown that both chondrogenic differentiation of neural crest cells and osteogenesis of neural crest-derived ectomesenchyme depend upon interactions of these cells with epithelial cells/ECM (7). Thorogood et al. (29) have demonstrated a transient expression of type II collagen at epitheliomesenchymal interfaces during formation of the chick cartilaginous neurocranium, which they hypothesize may act as a temporal and spatial signal in the ECM for overt chondrogenesis. In this context, the appearance of type VIII collagen might also be linked to that of type II (or other components of the ECM). Since we do not know whether type VIII collagen is expressed transiently or becomes incorporated into the ECM as a permanent structural element, the role of this protein in directing cellular migration or interactions cannot be ascertained at the present time.

The meninges of the central nervous system provide another example of tissue derived from neural crest that is enriched for type VIII collagen (other examples being DM, sclera, and calvarium). Although morphological resolution in these frozen sections is limited, it is possible that the apparent staining of the pia mater in the spinal cord could arise from the subadjacent glial membrane that is formed from the

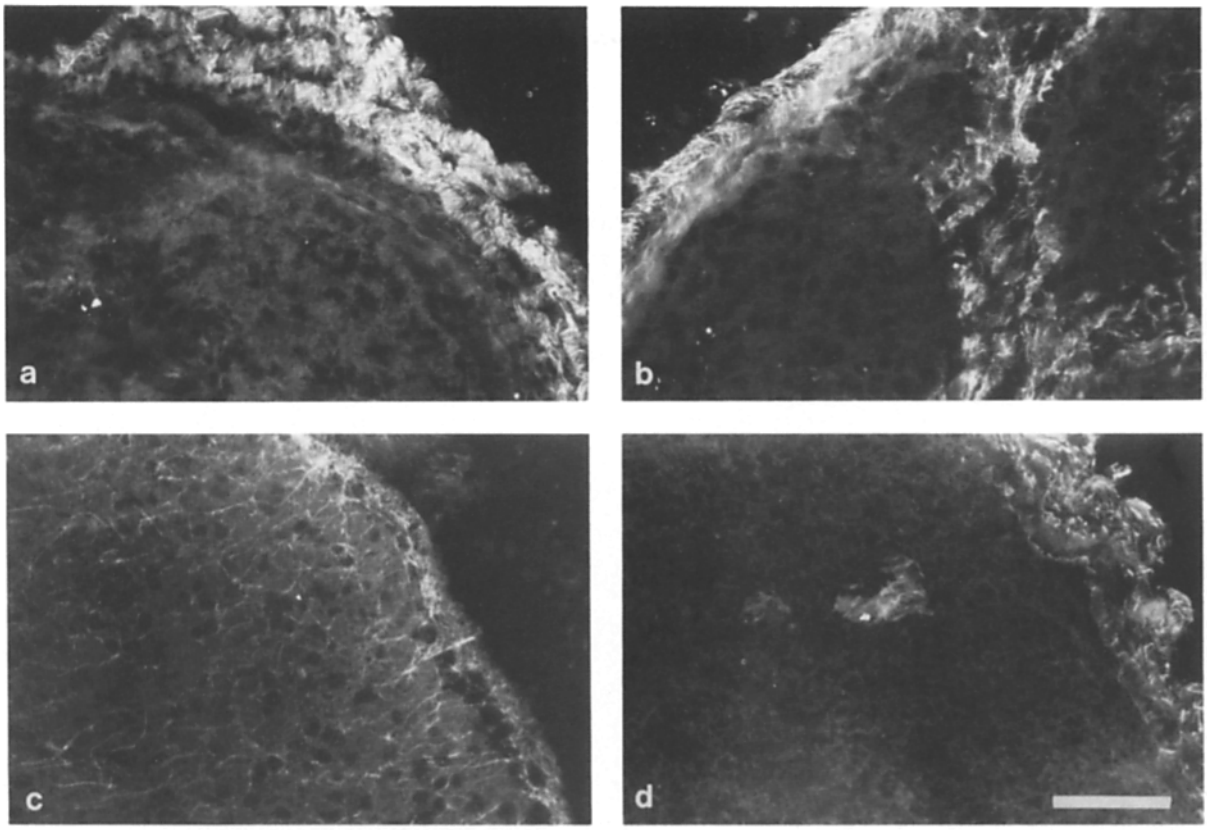

Figure 9. Immunofluorescence of type VIII collagen in nervous tissue. Frozen sections of fetal calf optic nerve (*a* and *b*) and spinal cord (*c* and *d*) were incubated sequentially with anti-type VIII collagen antibody (*a* and *c*) or anti-fibrillin antibody (*b* and *d*), and FITC anti-mouse IgG. Bar, 50 μm.

processes and terminal enlargements of fibrous astrocytes. This observation would be consistent with the heightened production of type VIII collagen by astrocytoma cells in culture (1). We have also found that cells derived from early stage and/or undifferentiated human astrocytomas produced type VIII collagen, while those from more advanced stages synthesized primarily the interstitial type I collagen (Sage, H., and L. Old, unpublished observations). Based on recent studies of glial cell progenitors in the developing rat optic nerve (28), we predict that type VIII collagen would be produced by, and possibly facilitate the migration of, type 2 astrocytes which contribute to the formation of the nodal axolemma.

In the adult, synthesis and assembly of type VIII collagen in the ECM may be related to tissue injury as previously suggested for other collagens and ECM components (13, 19). In accordance with conclusions derived from studies in which cell cultures were used (20), actively proliferating cells would preferentially synthesize type VIII collagen, while this gene would not be transcribed by quiescent cells in vivo. In the human cornea, there is a significant decline in the number of endothelial cells during development (~45% between 0 and 30 yr; 2). Morphologic studies on DM have revealed changes in collagen deposition concomitant with terminal differentiation and reorganization of the corneal endothelium around the time of birth (2). It is possible that type VIII collagen is a product of undifferentiated or stem cells and that the type VIII gene is reactivated during cellular proliferation as a result of injury. In this case, understanding the regulation of this collagen may reveal basic mechanisms underlying cellular differentiation.

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